Neutrophil Adherence to Bladder Microvascular Endothelial Cells following Platelet-Activating Factor Acetylhydrolase Inhibition

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ABSTRACT

Interstitial cystitis (IC) is an inflammatory bladder condition of unknown etiology. Tryptase released from elevated numbers of activated mast cells is a proposed mediator of the inflammatory process in IC. We have previously shown that tryptase increases human bladder microvascular endothelial cell (HBMEC) calcium-independent phospholipase A2 (iPLA2) activity, resulting in the production of multiple biologically active phospholipid metabolites, including platelet-activating factor (PAF), that can mediate inflammation. Because the design of selective PLA2 inhibitors may provide a useful therapeutic strategy to reduce the inflammatory process in IC, we tested several frequently used PLA2 inhibitors on PAF production in tryptase-stimulated HBMEC. Among the inhibitors tested, methyl arachidonyl fluorophosphonate (MAFP) was found to be a potent inhibitor of PAF-acetylhydrolase activity. Pretreatment of HBMEC with MAFP significantly increased PAF production in both unstimulated and tryptase-stimulated cells. In addition, MAFP pretreatment of tryptase-stimulated HBMEC increased both surface expression of P-selectin and polymorphonuclear leukocyte adherence to the HBMEC monolayer. These effects suggest that MAFP has a proinflammatory effect, irrespective of its ability to inhibit PLA2.

Interstitial cystitis (IC) is characterized by symptoms that include urinary frequency, urgency, and bladder and pelvic pain (for review, see Warren and Keay, 2002). The two main etiologic theories for IC are a defect in the cytoprotective layer of the bladder urothelium and an increase in numbers of activated mast cells that release chemical mediators and contribute to the pain, inflammation, and tissue damage of the bladder lining (Theoharides et al., 2001; Selo-Ojeme and Onwude, 2004). In a previous study, we showed that tryptase increases human bladder microvascular endothelial cell (HBMEC) calcium-independent PLA2 (iPLA2) activity, resulting in the production of multiple biologically active phospholipid metabolites that mediate the propagation of chronic inflammation (Rickard et al., 2004). These results suggest that inhibition of PLA2 activity in the bladder may provide a useful therapeutic avenue for the relief of IC.

PLA2-catalyzed hydrolysis of membrane phospholipids results in the stoichiometric production of a free fatty acid, most notably arachidonic acid, and a lysophospholipid. These phospholipid metabolites serve as precursors for inflammatory metabolites such as prostaglandins, leukotrienes, and platelet-activating factor (PAF). PAF is a highly potent phospholipid metabolite requiring concentrations as low as 10^{-12} M to exert its physiological effects (Stafforini et al., 2003). As such, the synthesis and degradation of PAF are tightly regulated within the cell. PAF is synthesized by endothelial cells in response to stimulation by a variety of agonists and may contribute to the progression of IC and other inflammatory disorders by increasing vascular permeability and the expression of endothelial cell surface adhesion molecules that facilitate transmigration of polymorphonuclear leukocytes (PMN) across the endothelial cell monolayer (Lefer and Lefer, 1996). The synthesis of PAF in endothelial cells occurs via the remodeling pathway, activated during inflammation and hypersensitivity responses, as opposed to the de novo synthesis of PAF.

INTERSTITIAL CYSTITIS (IC) IS AN INFLAMMATORY BLADDER CONDITION OF UNKNOWN ETIOLOGY. TRYPTASE RELEASED FROM ELEVATED NUMBERS OF ACTIVATED MAST CELLS IS A PROPOSED MEDiator OF THE INFLAMMATORY PROCESS IN IC. WE HAVE PREVIOUSLY SHOWN THAT TRYPTASE INCREASES HUMAN BLADDER MICROVASCULAR ENDOTHELIAL CELL (HBMEC) CALCIUM-INDEPENDENT PHOSPHOLIPASE A2 (iPLA2) ACTIVITY, RESULTING IN THE PRODUCTION OF MULTIPLE BIOLOGICALLY ACTIVE PHOSPHOLIPID METABOLITES, INCLUDING PLATELET-ACTIVATING FACTOR (PAF), THAT CAN MEDIATE INFLAMMATORY REACTION. BECAUSE THE DESIGN OF SELECTIVE PLA2 INHIBITORS MAY PROVIDE A USEFUL THERAPEUTIC STRATEGY TO REDUCE THE INFLAMMATORY PROCESS IN IC, WE TESTED SEVERAL FREQUENTLY USED PLA2 INHIBITORS ON PAF PRODUCTION IN TRYPTASE-STIMULATED HBMEC. AMONG THE INHIBITORS TESTED, METHYL ARACHIDONYL FLUOROPHOSPHONATE (MAFP) WAS FOUND TO BE A POTENT INHIBITOR OF PAF-ACETYLHYDROLASE ACTIVITY. PRETREATMENT OF HBMEC WITH MAFP SIGNIFICANTLY INCREASED PAF PRODUCTION IN BOTH UNSTIMULATED AND TRYPTASE-STIMULATED CELLS. IN ADDITION, MAFP PRETREATMENT OF TRYPTASE-STIMULATED HBMEC INCREASED BOTH SURFACE EXPRESSION OF P-SELECTIN AND POLYMORPHONUCLEAR LEUKOCYTE ADHERENCE TO THE HBMEC MONOLAYER. THESE EFFECTS SUGGEST THAT MAFP HAS A PROINFLAMMATORY EFFECT, IRRESPECTIVE OF ITS ABILITY TO INHIBIT PLA2.

ABSTRACT

Interstitical cystitis (IC) is a inflammatory bladder condition of unknown etiology. Tryptase released from elevated numbers of activated mast cells is a proposed mediator of the inflammatory process in IC. We have previously shown that tryptase increases human bladder microvascular endothelial cell (HBMEC) calcium-independent phospholipase A2 (iPLA2) activity, resulting in the production of multiple biologically active phospholipid metabolites, including platelet-activating factor (PAF), that can mediate inflammation. Because the design of selective PLA2 inhibitors may provide a useful therapeutic strategy to reduce the inflammatory process in IC, we tested several frequently used PLA2 inhibitors on PAF production in tryptase-stimulated HBMEC. Among the inhibitors tested, methyl arachidonyl fluorophosphonate (MAFP) was found to be a potent inhibitor of PAF-acetylhydrolase activity. Pretreatment of HBMEC with MAFP significantly increased PAF production in both unstimulated and tryptase-stimulated cells. In addition, MAFP pretreatment of tryptase-stimulated HBMEC increased both surface expression of P-selectin and polymorphonuclear leukocyte adherence to the HBMEC monolayer. These effects suggest that MAFP has a proinflammatory effect, irrespective of its ability to inhibit PLA2.

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ABBREVIATIONS: IC, interstitial cystitis; HBMEC, human bladder microvascular endothelial cell(s); iPLA2, calcium-independent phospholipase A2; PAF, platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine); PMN, polymorphonuclear leukocyte(s); lysoPlsEtn, lysophosphatidylethanolamine; PAF-AH, platelet-activating factor-acetylhydrolase; MAFP, methyl arachidonyl fluorophosphonate; cPLA2, cytosolic PLA2; BEL, bromoenoil lactone [(-)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one]; AACOCF3, arachidonyl trifluoromethyl ketone; PX-18, 2-[N,N-bis(2-oleoyloxyethyl)amino]-1-ethanesulfonic acid; MPO, myeloperoxidase; SEM, scanning electron microscopy; sPLA2, secreted phospholipase A2.
pathway in which PAF is synthesized for physiologic functions (Stafforini et al., 2003). In thrombin-stimulated endothelial cells, we have shown that the remodeling pathway begins with the activation of iPLA2, which catalyzes the hydrolysis of membrane plasmalogenethanolamine to yield lysoplasmenylethanolamine (lysoPlsEtn) and free arachidonic acid (Kell et al., 2003). The lysophospholipid then acts as an acceptor for an sn-2 fatty acid in a CoA-independent transacylation reaction with alkyl acyl glycerophosphorylcholine to generate lyso-PAF. Finally, lyso-PAF is acetylated at the sn-2 position in a reaction catalyzed by lyso-PAF acetyltransferase. The biological activities of PAF can be rapidly terminated by PAF-acetylhydrolyases (PAF-AH), a family of unique iPLA2 enzymes that hydrolyze the acetyl group at the sn-2 position of PAF to generate biologically inactive lyso-PAF and acetate.

The efficient degradation of PAF and the abrupt abrogation of its inflammatory actions have led to the characterization of PAF-AH as an anti-inflammatory enzyme (Tjoelker et al., 1995). In addition, evidence suggests that the inhibition of PAF-AH activity contributes to PAF-mediated proinflammatory effects. Deficiency in PAF-AH is the mechanism implicated in several pathological conditions and has been shown to be caused by a number of genetic mutations, each resulting in inactivation of PAF-AH activity (Stafforini et al., 1996; Tjoelker and Stafforini, 2000; Prescott et al., 2002). In animal studies, administration of recombinant human PAF-AH resolved many of the PAF-induced inflammatory effects (Henderson et al., 2000). These observations highlight the tight regulation of PAF bioactivities mediated by PAF-AH.

Much effort has gone into designing PLA2 inhibitors to be used therapeutically as anti-inflammatory agents. As more information about PLA2 isoforms has expanded, some PLA2 inhibitors originally thought to be selective for a single PLA2 isoform have been found subsequently to inhibit more than one isoform. For example, methyl arachidonoyl fluorophosphate (MAFP) was originally designed as a selective inhibitor of cytosolic PLA2 (cPLA2) and has subsequently been described as a potent and irreversible inhibitor of several PLA2 isoforms, including iPLA2 and PAF-AH (Lio et al., 1996; Bell et al., 2003). Furthermore, PLA2 inhibitors may inhibit other enzymes involved in membrane phospholipid hydrolysis or remodeling. For example, bromoeno lactone (BEL), a selective inhibitor of iPLA2, has been shown to inhibit phosphatidate phosphohydrolase (Zapan et al., 1993; Balsinde and Dennis, 1996a). Therefore, the development of a PLA2 inhibitor as a potential anti-inflammatory agent must undergo rigorous evaluation.

In the present study, we pretreated HBMEC with PLA2 inhibitors before stimulation with tryptase and measured their effect on PAF production. These data show that MAFP increases tryptase-stimulated PAF production because of its inhibition of PAF-AH and that this is associated with increased cell surface expression of P-selectin. Both of these responses can contribute to increased adherence of PMN to the endothelial cell monolayer.

Materials and Methods

Materials. Arachidonyl trifluoromethyl ketone (AACOCF3, an inhibitor of cPLA2 and iPLA2), BEL (a selective inhibitor of iPLA2), and MAFP were purchased from Cayman Chemical (Ann Arbor, MI). Recombinant human β-trypntase was purchased from Promega (Madison, WI). Richard Berney (Richard Berney Associates, LLC, Bethesda, MD) donated PX-18. [3H]Acetic acid, [14C]Acetic anhydride, and [acetly-3H]PAF were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Culture. HBMEC were grown in EGM-2MV medium (Cambrex Bio Science Baltimore, Inc., Baltimore, MD) and maintained at 37°C in a humidified atmosphere of 95% air and 5% CO2. We extensively characterized these cells in a previous publication (Richard et al., 2004).

PAF Production. HBMEC grown to confluence were incubated with Hanks’ balanced salt solution (135 mM NaCl, 0.8 mM MgSO4, 10 mM HEPES, pH 7.4, 1.2 mM CaCl2, 5.4 mM KCl, 0.4 mM KH2PO4, 0.3 mM Na2HPO4, and 6.6 mM glucose) containing 10 μCi of [3H]Acetic acid for 20 min at room temperature. Following incubation with 5 μM MAFP, BEL, or PX-18 for 10 min, cells were incubated with 20 ng/ml of tryptase or 5 μM lysoPlsEtn for an additional 10 min. Each incubation was terminated by the addition of methanol, and the cells were removed from the tissue culture well. Total lipids were extracted from the cells according to the method of Bligh and Dyer (1959). The chloroform layer was concentrated by evaporation under N2. Isolated lipids were resuspended in a 9:1 v/v mixture of chloroform and methanol, applied to silica gel plates, and developed in a 50:25:8:4 v/v mixture of chloroform, methanol, glacial acetic acid, and water. The region corresponding to PAF was scraped off the plate, and incorporated radioactivity was measured using a liquid scintillation counter. [14C]PAF was used as an internal standard to correct for the loss of PAF during the experimental procedure as described previously (McHowat et al., 2001).

PAF-AH Activity. PAF-AH activity was measured as described previously (McHowat et al., 2001). Briefly, HBMEC grown to confluence were incubated with the appropriate PLA2 inhibitors for the required time, then removed from the tissue culture plate in 1.2 mM Ca2+ HEPES buffer, and sonicated on ice. Cellular protein (25 μg) was incubated with 0.1 mM [acetly-3H]PAF (10 μCi/mmol) for 30 min at 37°C. The reaction was stopped by the addition of acetic acid and sodium acetate. Released [3H]Acetic acid was isolated by passing the reaction mixture through a C18 silica gel column (J. T. Baker, Phillipsburg, NJ), and eluted radioactivity was measured using a liquid scintillation counter.

P-Selectin Surface Expression Assay. HBMEC were grown to confluence in 16-mm culture dishes. Cultures, including controls and those pretreated for 10 min with MAFP (5 μM), BEL (2 μM), or PX-18 (2 μM), were incubated with or without tryptase (20 ng/ml) for an additional 10 min. The medium was quickly removed and replaced with a solution containing 127 mM NaCl, 5 mM KCl, 1.1 mM NaH2PO4, 0.4 mM KH2PO4, 2 mM MgCl2, 5.5 mM glucose, 20 mM PIPES, and 1% paraformaldehyde, pH 6.5. Fixation was performed at 4°C overnight. After washing three times with phosphate-buffered saline, the cells were incubated in Tris-buffered saline containing 0.1% w/v Tween, 0.8% w/v bovine serum albumin, and 0.5% w/v fish gelatin for 1 h at 24°C. The cells were incubated with anti-P-selectin goat polyclonal antibody (1:50 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), followed by incubation with horseradish peroxidase-conjugated rabbit anti-goat secondary antibody. After washing, each culture dish was incubated in the dark with 3,3',5,5'-tetramethylbenzidine liquid substrate for 1 h. Reactions were terminated by the addition of 8 N sulfuric acid. Color development was measured with a microtiter plate spectrophotometer at 450 nm.

PMN Isolation and Adherence Assay. Human PMN were isolated from the peripheral blood of one healthy adult per experiment following dextran sedimentation on Polymorphprep solution (Axis-Shield, Oslo, Norway). After centrifuging at 400g for 30 min, the layer of PMN was removed, and contaminating red blood cells were
lysed using 0.2% NaCl. Cell viability was determined to be >90% based on exclusion of 0.2% trypan blue. PMN were washed with Hanks' buffer and resuspended in Dulbecco's minimal essential medium supplemented with 20% bovine serum. Confluent HBMEC monolayers in 34-mm culture dishes were washed twice with Dulbecco's minimal essential medium. PMN (2 × 10^6) were added to each dish, and control or PLA2 inhibitor-treated cells were stimulated with ± tryptase (20 ng/ml) for 10 min.

At the end of stimulation, nonadherent PMNs were removed by washing the well twice with Hanks' buffer. After 0.2% Triton X-100 was added to each well to lyse the HBMEC and adherent PMN, the lysate was removed and sonicated on ice for 6 s. The myeloperoxidase (MPO) content of the adherent PMN was determined by adding 400 μl of cell lysate to a tube containing 1 ml of PBS, 1.2 ml of Hanks' buffer with bovine serum albumin, 200 μl of 0.125% 3,3'-dithiobis (55-fluorobenzoic acid) benzine, and 200 μl of 0.05% H_2O_2. After samples were incubated at 37°C for 15 min, the reaction was stopped by the addition of 200 μl of Na_2S_2O_3, and the absorbance of each tube was measured at 460 nm. MPO content in 2 × 10^6 PMN was determined and used as the value for 100% adherence.

Microscopic Examination of Endothelial Cell-PMN Interaction. HBMEC were grown to confluence on transwell inserts and processed using standard procedures as described in detail previously (Ryser, 1989; Farberman et al., 2005). Briefly, the cells were fixed with glutaraldehyde, postfixed with osmium tetroxide, dehydrated through graded ethanols, and embedded in PolyBed (Polysciences, Warrington, PA) resin for transmission electron microscopy or critical point dried and coated with gold-palladium in a sputter coater for scanning electron microscopy (SEM). Thick (0.25 μm) plastic sections were stained with toluidine blue for light microscopy, and thin (50–60 nm) sections were stained with uranyl acetate and lead citrate for transmission electron microscopy. Light micrographs were taken with an Olympus (Tokyo, Japan) digital camera; thin sections were viewed and photographed on a JEOL (Tokyo, Japan) 100CX EM; and the SEM samples were viewed and photographed with a JEOL 5800 SEM.

Results

To investigate the role of PLA2 isoforms in the production of PAF in response to short intervals of tryptase stimulation, we pretreated HBMEC with MAPF (an inhibitor of cytosolic PLA2 isoforms, 5 μM, 10 min) (Huang et al., 1994; Lio et al., 1996), PX-18 [a selective secretory phospholipase A2 (sPLA2) inhibitor, 5 μM, 10 min] (Franson et al., 1991; Franson and Rosenthal, 1997), or BEL (a selective iPLA2 inhibitor, 5 μM, 10 min) (Zupan et al., 1993) before tryptase stimulation (20 ng/ml, 10 min). Pretreatment with BEL resulted in no change in PAF production in unstimulated HBMEC but completely inhibited tryptase-stimulated PAF production as shown previously (Fig. 1) (Rickard et al., 2004). Pretreatment with PX-18 partially inhibited tryptase-stimulated PAF production, whereas pretreatment with MAPF significantly potentiated PAF production in both unstimulated and tryptase-stimulated cells (Fig. 1).

To determine whether MAPF inhibits PAF-AH in HBMEC, we measured PAF-AH activity in MAPF-treated cells. We found that pretreatment with MAPF resulted in a concentration-dependent inhibition of PAF-AH activity having an IC_{50} of 0.3 μM (Fig. 2). This is similar to the concentrations of MAPF that inhibit cPLA2 isoforms (Balsinde and Dennis, 1996b). At higher concentrations of MAPF (10 μM), PAF-AH activity is almost completely abrogated (Fig. 2). The results with MAPF significantly contrast those obtained with pretreatment of HBMEC with BEL, PX-18, or the reversible cPLA2 and iPLA2 inhibitor, AACOCF_3 (Ackermann et al., 1995), which did not have a significant effect on PAF-AH activity even when used at higher concentrations (10 μM; Fig. 2). We also stimulated HBMEC with up to 200 ng/ml of
tryptase and observed that PAF-AH activity was not significantly affected (data not shown). These results show that MAPF potentiates the production of PAF by a mechanism that involves inhibition of PAF hydrolysis by PAF-AH.

To determine whether the remodeling pathway is involved in PAF production in HBMEC similar to that observed in previous endothelial cell experiments (McHowat et al., 2001), we pretreated cells with MAPF before tryptase or lysoPlsEtn stimulation. Incubation of HBMEC with tryptase or lysoPlsEtn resulted in similar increases in PAF production that were significant in comparison to unstimulated cells (Fig. 3, filled columns). Pretreatment with MAPF significantly increased PAF production in unstimulated, tryptase-stimulated, and lysoPlsEtn-stimulated HBMEC (Fig. 3, open columns). These results show that in HBMEC, the majority of PAF synthesized in response to tryptase stimulation is through the remodeling pathway.

Assessment of cell surface expression of P-selectin using spectrophotometric analysis showed a basal expression of P-selectin on unstimulated HBMEC (Fig. 4). Pretreatment with MAPF in unstimulated or tryptase-stimulated cells resulted in significant increases in absorbance compared with cells not pretreated with MAPF, indicating enhanced cell surface P-selectin expression. In contrast, pretreatment with BEL or PX-18 significantly inhibited the tryptase-induced cell surface expression of P-selectin (Fig. 4). To assess whether MAPF-dependent upregulation of P-selectin cell surface expression and increased PAF production resulted in enhanced PMN adherence, we pretreated HBMEC with MAPF, followed by stimulation with tryptase (20 ng/ml), and incubated with PMN. Adherence of leukocytes was quantified using the MPO assay as described under Materials and Methods. In unstimulated or tryptase-stimulated HBMEC pretreated with MAPF, there was approximately a 2-fold increase in the number of adherent PMN (Fig. 5). Pretreatment with BEL or PX-18 inhibited PMN adherence in tryptase-stimulated HBMEC. These results show that MAPF potentiates both PAF production and P-selectin cell surface expression resulting in increased leukocyte adherence to the endothelial cell monolayer.

Microscopy was used to show that neutrophils make contact with endothelial cells growing on a transwell membrane (Fig. 6). A confluent layer of HBMEC was incubated with 1 × 10⁶ PMN and MAPF (5 μM, 10 min) before processing for microscopy as described above. Neutrophils were observed adhering to the HBMEC monolayer in toluidine blue-stained thick plastic sections (Fig. 6A) and in samples processed for SEM (Fig. 6B). The neutrophils have cell surface ruffles and lamellipodia extending along the endothelial cell surface, both indicators of activated, motile leukocytes (Farberman et al., 2005). Transmission electron microscopy clearly shows that there is close apposition and cell contact between neutrophils and endothelial cells (Fig. 6C). The neutrophils develop extensions that seem to probe endothelial cells and make cell surface contact.

**Discussion**

PAF production is tightly regulated by its synthesis (mediated by iPLA₂) and degradation (mediated by PAF-AH). PAF-AH inactivates PAF by catalyzing the removal of the acetyl group at the sn-2 position of its glycerol backbone (Stafforini et al., 1997). We have shown that MAPF potentiates the production of PAF (Fig. 1) by inhibiting PAF-AH.
activity in a concentration-dependent manner (Fig. 2). This property seems unique to MAFP because pretreatment of HBMEC with other PLA2 inhibitors tested did not affect PAF-AH activity.

PAF production in endothelial cells proceeds primarily via the remodeling pathway in which the initial step involves iPLA2-catalyzed hydrolysis of plasmenylethanolamine. This initial reaction produces lysoPlsEtn that acts as an acyl group acceptor in a transacylation reaction to produce lysoPAF, which is finally acetylated to produce PAF. We have previously shown that PAF production proceeds via the remodeling pathway in thrombin-stimulated human umbilical artery endothelial cells (McHowat, 2001). The increase in PAF production after incubation with lysoPlsEtn in this study shows the involvement of the remodeling pathway for PAF synthesis in HBMEC. The inhibition of PAF-AH with MAFP resulted in a significant potentiation of PAF production after both tryptase and lysoPlsEtn stimulation.

MAFP is an active site-directed, irreversible inhibitor of cPLA2 and iPLA2. These enzymes do not require Ca2+/H2O1 for catalytic activity, use a central serine for catalysis, and operate via the formation of an acyl-enzyme intermediate (Lio et al., 1996; Six and Dennis, 2000). Our results showing that MAFP inhibits PAF-AH suggest that MAFP may be a relatively nonselective inhibitor of multiple serine-dependent lipases. PAF-AH, which comprises the group VII and VIII iPLA2 enzymes, resembles many neutral lipases in that a Ser-His-Asp triad characterizes the mechanism of catalysis (Tjoelker et al., 1995). In contrast, other PLA2 inhibitors used in this study had no effect on PAF-AH activity. BEL is an active site-directed, irreversible inhibitor of group VI iPLA2 (Zupan et al., 1993). PX-18 is a recently developed reversible inhibitor that shows selectivity for sPLA2 (Franson et al., 1991; Franson and Rosenthal, 1997). AACOCF3, a trifluoromethyl ketone analog of arachidonic acid, is a reversible inhibitor of both cPLA2 and iPLA2 isoforms (Ackermann et al., 1995). The mechanism of inhibition by AACOCF3 is similar to MAFP in that both compounds compete with the endogenous phospholipid molecule for the active catalytic site on the PLA2 enzymes.

The leukocyte-endothelial interaction involves surface expression of adhesion molecules that tether PMN to the endothelial cell membrane (Zimmerman et al., 1992). On stimulation by inflammatory mediators, including histamine and thrombin, Weibel-Palade bodies containing intracellular stores of P-selectin rapidly translocate to the plasma membrane and release their contents to expose P-selectin on the endothelial cell surface (McEver et al., 1989; Geng et al., 1990). In thrombin-stimulated endothelial cells, PAF is another proadhesive molecule that is coexpressed at the cell surface.
surface with P-selectin (Lorant et al., 1991). These two molecules act cooperatively to initiate a cascade of events that results in firm PMN adhesion and eventually to leukocyte extravasation. Endothelial cell surface expression of P-selectin mediates PMN rolling along the endothelium allowing for the newly synthesized PAF to interact with its receptor on the PMN and to signal upregulation of CD11/CD18 glycoproteins on the PMN plasma membrane. The induction of intercellular adhesion molecule-1 on the endothelium together with PMN CD11/CD18 expression supports firm leukocyte adherence to the endothelium. Finally, endothelial platelet-endothelial cell adhesion molecule-1 cell surface expression mediates effective PMN transmigration to extravascular sites (Lorant et al., 1991; Lefer and Lefer, 1996). In this study, we found that MAFP can stimulate both PAF synthesis and cell surface expression of P-selectin. Furthermore, we showed that in both unstimulated and tryptase-stimulated HBMEC, pretreatment with MAFP produced significant increases in PMN adherence.

It is of interest that MAFP induced the synthesis of PAF and cell surface expression of P-selectin in HBMEC concurrently. This observation agrees with recent work suggesting that in activated endothelial cells, the signal transduction mechanisms regulating PAF and P-selectin are interrelated. Rollin et al. (2004) showed that the synthesis of PAF is essential for the cell surface expression of P-selectin when endothelial cells were stimulated with vascular endothelial growth factor. In addition, studies in platelets indicate that cell surface expression of P-selectin is induced by exposure of the cells to PAF after the activation of at least three intracellular signal transduction pathways, including protein tyrosine phosphorylation, Na+/H+ exchange, and Ca2+ mobilization (Zeng et al., 1999). Thus, the evidence published to date suggests that the production of PAF and the cell surface expression of P-selectin are concurrent events that are dependent on similar intracellular pathways.

These data highlight the necessity for highly specific PLA2 inhibitors in order for them to be used therapeutically as anti-inflammatory agents. Although several causative factors have been proposed for IC, the etiology is unknown. In a recent review, possible causative considerations were infections, immunologic and neurogenic responses, and a defective glycosaminoglycan layer in the bladder (Nickel, 2004). Furthermore, IC may not be a single disease entity in all the patients based on the evidence of IC subtypes and its comorbidity with other unexplained clinical conditions (Buffington, 2004). IC is characterized by a defect in bladder cytoprotection by the urothelium and a pathophysiologic role for the mast cell (Theoharides et al., 2001). Whether this is because of the compromise in barrier function of the urothelium that allows released mast cell activators to penetrate the bladder wall and perpetuate inflammation remains to be clarified.

Because of the numerous etiologies for IC, the focus of current medical therapies is to provide relief of symptoms and to increase the overall quality of life for the patient. A potentially beneficial target for the treatment of IC and other inflammatory diseases is the use of pharmacological PLA2 inhibitors that could be instilled into the bladder or concentrated in the urine, hence minimizing potential systemic side effects (Meyer et al., 2005). The use of these agents would inhibit the production of several membrane phospholipid-derived metabolites that play a role in inflammation, including lysoplasmalogen, PAF, and arachidonic acid and its metabolites. To date, the only designed inhibitors to have advanced into clinical trials are selective for the sPLA2 enzymes (Tykka et al., 1985; Abraham et al., 2003; Scott et al., 2003). We showed in Fig. 1 that incubation of HBMEC with PX-18 resulted in a significant decrease in tryptase-stimulated PAF production of ~50%, suggesting that sPLA2 may also be involved in endothelial PAF production, although iPLA2 seems to be the principal isoform that contributes significantly to the synthesis of PAF as evidenced by the complete inhibition of PAF production by BEL. This effect of PX-18 is independent of iPLA2 activity because we did not measure a significant inhibition of iPLA2 activity in the cytosolic (1.2 ± 0.1 versus 1.3 ± 0.1 nmol/mg protein/min, n = 6) or membrane fractions (6.1 ± 0.7 versus 6.7 ± 0.8 nmol/mg protein/min, n = 6) of HBMEC following PX-18 treatment (5 μM, 10 min). As more information becomes available regarding the role of PLA2 enzymes in different organs and cells, our ability to custom design inhibitors for a single disease process is enhanced. There are relatively few data available that highlight the role of PLA2 in the bladder in inflammatory diseases such as IC, but PLA2 inhibitors may prove to be useful in alleviating the morbidity of this disease.

In summary, we have shown that in HBMEC, MAFP potentiates PAF production in both unstimulated and tryptase-stimulated cells by inhibiting PAF-AH activity. Further, we have shown that MAFP augments cell surface P-selectin expression and PMN adherence to the endothelial monolayer in unstimulated and tryptase-stimulated cells. However, pretreatment with BEL or PX-18 significantly inhibited PAF production, cell surface expression of P-selectin, and PMN adherence to the HBMEC monolayer in response to tryptase stimulation. These data suggest that there may be an interaction between iPLA2 and sPLA2 in tryptase-stimulated HBMEC that results in increased PAF production. In addition, inhibition of HBMEC iPLA2 with BEL or sPLA2 with PX-18 may represent a potential anti-inflammatory therapeutic avenue in IC.

PAF signaling is terminated by the rapid degradation by PAF-AH, thereby providing an immediate defense mechanism to prevent proinflammatory effects. In endothelial cells, the dysregulation of this mechanism would lead to the diminished ability to terminate the biological activities of PAF, resulting in accumulation of PAF and subsequent PMN activation. Thus, maintaining appropriate PAF-AH activity is essential to suppress the effects of PAF in active inflammation. Our data using MAFP highlight the key role of PAF-AH in the regulation of PAF-induced inflammatory responses in bladder endothelium.

References


Phospholipase A2 inhib.


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